

REMARKS

Claim 27 is amended herein to delete reference to an "extracellular domain" and claims 30-31 are deleted herein solely to expedite prosecution and not for reasons related to patentability. Claim 27 is amended and claims 30-31 are cancelled without disclaimer or prejudice to pursuing the full invention of claims 27 and 30-31 in continuing applications.

Claims 27-29, and 32-34 are pending in this application.

Applicants thank the Examiner for withdrawing the previous rejection of claims 27-34 under 35 U.S.C. § 101 because the claimed invention was allegedly not supported by either a specific and substantial utility or a well-established utility.

Applicants also thank the Examiner for withdrawing the previous rejection of claims 27-34 under 35 U.S.C. § 112, first paragraph because one of ordinary skill in the art would allegedly not know how to use the claimed invention.

Applicants respectfully traverse the new rejections and request reconsideration in light of the below submitted evidence and arguments.

New Rejection under 35 U.S.C. §§ 101, 112, First Paragraph

Claims 27-34 are rejected under 35 U.S.C. § 101 because allegedly the claimed invention is not supported by either a credible, specific, and substantial asserted utility or a well established utility. Claims 27-34 are also rejected under 35 U.S.C. § 112, first paragraph because allegedly the claimed invention is not supported by an adequate utility and thus, allegedly one of ordinary skill in the art would not know how to use the claimed invention.

Applicants thank the Examiner for noting that a portion of the previous rejection is withdrawn. Specifically, the Examiner no longer asserts that mRNA levels are not predictive of polypeptide levels. Thus, the following references are overcome: Haynes, Gygi, Llan, Fessler, LaBaer, Chen, Hanna, Greenbaum, Winstead, Irving, etc. The Examiner further notes that the following references and evidence, cited and relied on by Applicants, will no longer be addressed: Scott Declaration, Polakis Declarations, Futch et al, Alberts and Lewin, Meric, Zhigang etc.

According to the Office action, the basis of the instant rejection is solely that gene amplification levels are not predictive of mRNA or polypeptide levels. However, as an initial matter, the Office action alleges that because only 12 out of 27 colon cancer samples tested positive, if a sample were taken from an individual with colon cancer for diagnosis, it is more likely than not that this assay would yield a false negative result.

Applicants respectfully disagree that this indicates the present claims are not supported by an adequate utility. For utility, PRO357 does not have to be amplified in every incidence of colon cancer. Indeed, there is no requirement that the claimed PRO357 polypeptide identify all types and cases of cancer. As the Office action acknowledges, the nucleic acid encoding PRO357 is amplified in 93% of the lung tumor samples tested. Thus, Applicants respectfully submit that the gene amplification data provides a sufficient utility, at least with respect to a diagnostic in lung tumor tissue. A single utility is sufficient to satisfy the utility requirement.

Moreover, any skilled artisan in the field of oncology would appreciate that not all tumor markers are generally associated with every tumor, or even, with most tumors. In fact, some tumor markers are useful for identifying rare malignancies. That is, the association of the tumor marker with a particular type of tumor lesion may be rare, or, the occurrence of that particular kind of tumor lesion itself may be rare. In either event, even these rare tumor markers, which may not give a positive hit with most common tumors, have great value in tumor diagnosis and consequently, in tumor prognosis. The skilled artisan would know that such tumor markers are very useful for better classification of tumors. Therefore, whether the PRO357 gene is amplified in most of the colon tumors is not relevant to its identification as a tumor marker, or its patentable utility.

Next the Office action alleges that "even if a majority of lung and colon tumor samples had tested positive, the data has no bearing on the utility of the claimed PRO357 polypeptides . . . [because] for PRO357 polypeptides to be overexpressed in tumors, amplified genomic DNA would have to correlate with increased mRNA levels and increased polypeptide levels." Page 5 of the Office action mailed 1/22/08. The Office action alleges that the art discloses that a correlation cannot be presumed. In support, the Office action relies on

references by Pennica, Konopka, Godbout, and Li. However, as discussed below, these references do not outweigh the references relied on by Applicants and do not make it more likely than not that gene amplification does not correlate with mRNA or polypeptide overexpression.

As a preliminary matter, Applicants respectfully note that it is not a legal requirement to establish that gene amplification “necessarily” results in increased expression at the mRNA and polypeptide levels or that polypeptide levels can be “accurately predicted.” As discussed in previous responses, the evidentiary standard to be used throughout *ex parte* examination of a patent application is a preponderance of the totality of the evidence under consideration. Therefore, to overcome the presumption of truth that Applicants’ assertion of utility enjoys, the Office action must establish that **it is more likely than not that one of ordinary skill in the art would doubt Applicants’ assertion of utility**. Applicants respectfully submit that when this proper standard is applied, correlation between gene amplification and levels of mRNA or polypeptide expression must be acknowledged.

Orntoft, Pollack, Hyman, Jares, Fan, Saretzki, Sohn, Forus, Walch, de la Guardia, Walker, Blancato, and Cancer Medicine evidence that gene amplification more likely than not correlates with mRNA and polypeptide overexpression:

As the Office action notes, Applicants maintain that Orntoft, Pollack, and Hyman demonstrate that gene amplification levels are more likely than not to correlate with mRNA levels. Indeed, Applicants maintain that these references support Applicants assertion of utility.

Orntoft *et al.* studied transcript levels of 5600 genes in malignant bladder cancers, and found that in general (18/23 cases) chromosomal areas with more than 2-fold gain of DNA showed a corresponding increase in mRNA transcripts. Orntoft *et al.* also showed a clear correlation between mRNA and protein expression levels, stating that “[i]n general there was a highly significant correlation ($p<0.005$) between mRNA and protein alteration . . . 26 well focused protein whose genes had a known chromosomal location were detected in TCCs 733 and 335, and of these 19 correlated ($p<0.005$) with the

mRNA changes detected using the arrays." (See page 42, column 2, to page 44, column 2). Accordingly, Orntoft *et al.* clearly support Applicants' position that proteins expressed by genes that are amplified in tumors are useful as cancer markers.

Similarly as previously argued, Hyman *et al.* compared DNA copy numbers and mRNA expression of over 12,000 genes in breast cancer tumors and cell lines, and found that there was evidence of a prominent global influence of copy number changes on gene expression levels.

In Pollack *et al.*, the authors profiled DNA copy number alteration across 6,691 mapped human genes in 33 predominantly advanced primary breast tumors and 10 breast cancer cell lines, and found that on average, a 2-fold change in DNA copy number was associated with a corresponding 1.5-fold change in mRNA levels.

Numerous other art references support Applicants' assertion of utility. For example, Jares *et al.* teach that "[a] significant association was observed between gene amplification and mRNA overexpression [of *PRAD-1*, a putative oncogene localized on chromosome 11q13 which encodes cyclin D1] (P<0.0001) with only 3 discordant cases (2 amplifications with no overexpression and 1 overexpressed carcinoma with no gene amplification). Furthermore, the degree of DNA amplification correlated with the levels of mRNA expression ($r=0.6$; $P = 0.024$)."*Jares, et al., "PRAD-1/Cyclin D1 Gene Amplification Correlates with Messenger RNA Overexpression and Tumor Progression in Human Laryngeal Carcinomas," Cancer Research, 1994. 54:4813-4817 (Abstract).* (Tab A). Jares concludes that "*PRAD-1/cyclin D1* gene is amplified in a high number of laryngeal carcinomas and this amplification is significantly associated with mRNA overexpression." *Id.* at 4186.

Fan *et al.* report similar results stating, "[t]o correlate gene amplification with message level, we used real-time quantitative PCR to measure hTERT mRNA in 50 embryonal brain tumors. hTERT mRNA was detected in all but one of these cases, and mRNA level correlated significantly with gene dosage ($r=0.82$). *Fan et al., "hTERT Gene Amplification and Increased mRNA Expression in Central Nervous System Embryonal Tumors," Am. J. Path., 2003. 162(6):1763-1769 (Abstract).* (Tab B). Similar to Jares,

Fan concludes, "we have shown that the *hTERT* oncogene is amplified in a significant proportion of medulloblastomas and other CNS embryonal neoplasms. This gene amplification correlates with increased expression of *hTERT* mRNA. *Id.* at 1768. See also Saretzki *et al.*, "hTERT gene dosage correlates with telomerase activity in human lung cancer cell lines." *Cancer Lett.*, 2002. 176(1):81-91 ("We found a significant correlation between *hTERT* gene dosage, *hTERT* mRNA expression and telomerase activity.") (Tab C).

Sohn *et al.*, teach that the results of their cervical cancer study "indicate that the gene amplification and expression of MPG [N-methylpurine-DNA glycosylase] were increased in high-risk HPV-infected cervical neoplasias." Sohn *et al.*, "Gene amplification and expression of the DNA repair enzyme, N-methylpurine-DNA glycosylase (MPG) in HPV-infected cervical neoplasias," *Anticancer Res.*, 2001. 21 (4A):2405-11. (Tab D).

Forus teaches "PRUNE [the human homologue of the *Drosophila* gene] amplification was generally accompanied by high mRNA and moderate to high protein levels." Forus *et al.*, "Amplification and overexpression of PRUNE in human sarcomas and breast carcinomas-a possible mechanism for altering the nm23-H1 activity," *Oncogene*, 2001. 20(47):6881-90. (Tab E).

Walch reported similar findings in breast cancer: "Her-2/neu gene amplification was observed in 35% of BCA, and all of these samples showed strong overexpression of both mRNA and oncoprotein." Walch, *et al.*, "Her-2/neu gene amplification, elevated mRNA expression, and protein overexpression in the metaplasia-dysplasia-adenocarcinoma sequence of Barrett's esophagus," *Lab Invest.*, 2001. 81(6):791-801. (Tab F). See also, Walker *et al.*, "An immunohistochemical and in situ hybridization study of c-myc and c-erbB-2 expression in primary human breast carcinomas," *J. Pathol.* 1989. 158(2):97-105. ("There was a good correlation between gene amplification the presence of c-erbB-2 protein and mRNA: both the latter were detected in six of the seven carcinomas with an amplification but in none without.") (Tab G); Blancato *et al.*, "Correlation of amplification and overexpression of the c-myc oncogene in high-grade breast cancer: FISH, in situ hybridization and immunohistochemical

analyses," *Br. J. Cancer*, 2004. 90(8):1612-9 ("Statistically significant correlations were identified among the gene amplification indices, the RNA expression scores and protein expression scores. C-myc gene amplification, as detected by FISH, was significantly associated with expression of its mRNA."). (Tab H).

Cancer Medicine teaches "[i]n general, there is a correlation between MYCN copy number and expression at the messenger ribonucleic acid (mRNA) and protein levels." (Tab I). See also *de la Guardia, et al.*, "CENP-F gene amplification and overexpression in head and neck squamous cell carcinomas," *Head Neck*, 2001. 23(2):104-12 ("CENP-F gene is amplified and overexpressed in HNSCC." (Tab J).

Applicants respectfully submit that the aforementioned references relied on by Applicants demonstrate that more likely than not, the claimed invention is supported by a specific, and substantial diagnostic utility. Specifically, the utility is substantial because it distinguishes cancer cells from normal cells, which is not an insubstantial or trivial utility without a real world use. This utility is also specific because it is directed to specific types of cancer, lung and colon, and it is not a utility shared by every nucleic acid.

Pennica, Konopka, Godbout and Li do not outweigh the above-discussed evidence:

As stated previously, the Office action relies on references by Pennica, Konopka, Godbout and Li. The teachings of these references do not overcome the clear teaching of the above-discussed references, that generally, it is more likely than not that a gene amplified in cancerous tissues is overexpressed as mRNA and useful as a diagnostic.

Specifically, according to the Office action, Pennica *et al.*, provide "[a] specific example of the lack of correlation between genomic DNA amplification and increased mRNA expression." Page 6 of the Office action mailed 1/22/08. Applicants respectfully disagree that the teachings of Pennica demonstrate that *more likely than not* one of ordinary skill in the art would not expect gene amplification levels to correlate with protein overexpression. First, *WISP-1* gene amplification and RNA expression levels

examined in Pennica showed a significant positive correlation. Second, although Pennica stated that *WISP-3* was not significantly amplified, it was amplified ($P=1.666$) and overexpressed. Third, although *WISP-2* gene amplification and RNA expression levels seemed to be inversely related, Pennica suggests that this result might be inaccurate: “[b]ecause the center of the 20q13 amplicon has not yet been identified, it is possible that the apparent amplification observed for *WISP-2* may be caused by another gene in this amplicon.” See Pennica at 14722. Thus, because the RNA expression pattern of *WISP-2* cannot be accurately attributed to gene amplification of *WISP-2*, this result should be disregarded.

Although the Examiner correctly concludes that Pennica teaches that increased copy number does not *necessarily* result in increase polypeptide expression, the standard for utility is not absolute certainty. Thus, the fact that in the case of a specific class of closely related molecules there seemed to be no strong correlation with gene amplification and the level of mRNA/protein expression, does not establish that it is more likely than not, in general, that such correlation does not exist. The Examiner has not shown whether the lack of correlation observed for the family of WISP polypeptides is typical, or is merely a discrepancy, an exception to the rule of correlation. Indeed, the working hypothesis among those skilled in this art is that, if a gene is amplified in cancer, the encoded protein is likely to be expressed at an elevated level. In fact, as noted even in Pennica, “[a]n analysis of *WISP-1* gene amplification and expression in human lung tumors showed a correlation between DNA amplification and overexpression . . .” (Pennica, page 14722, left column, first full paragraph, emphasis added).

Accordingly, Applicants respectfully submit that Pennica teaches nothing conclusive regarding the absence of correlation between amplification of a gene and overexpression of the encoded WISP polypeptide. More importantly the teaching of Pennica et al is specific to *WISP* genes. As the PTO recognized during prosecution of US Patent No. 7,208,308 to Genentech, Pennica has no teaching whatsoever about the correlation of gene amplification and protein expression in general.

Applicants respectfully submit that the Examiner's reliance on Konopka is similarly misplaced. From that reference, the Examiner has generalized a very specific result to cover all genes. Konopka actually states that "[p]rotein expression is not related to amplification of the *abl* gene but to variation in the level of *bcr-abl* mRNA produced from a single Ph¹ template." (See Konopka, Abstract). This paper does not teach anything whatsoever about the correlation of protein expression and gene amplification in general, and provides no basis for the generalization that apparently underlies the present rejection. This statement of Konopka is not sufficient to establish a *prima facie* case of lack of utility. Indeed, just as the PTO recognized during prosecution of US Patent No. 7,208,308 that Pennica has no general teaching, the PTO also recognized that Konopka is not directed towards genes in general but rather to single genes or single families of genes. Thus, Konopka, like Pennica, cannot support a general conclusion against correlation of gene amplification and mRNA or polypeptide expression levels.

The Office action notes that another reference, Godbout, teaches that amplified genes are overexpressed if they provide a selective advantage. According to the Examiner, this reference demonstrates Applicants' asserted utility is insufficient because "[t]here is no evidence that PRO357 confers any growth advantage to a cell, and thus it cannot be presumed that the protein is overexpressed because the genomic DNA including the gene being studied is amplified." Page 7 of the Office action mailed 1/22/08.

As an initial matter, Applicants respectfully submit that it was never claimed that PRO357 is similar in any way to the DDX1 gene of Godbout. Applicants never claimed PRO357 was an RNA helicase or that it confers selective advantage to cell survival. Rather, the Godbout reference was submitted to show good correlation between protein levels and genomic DNA amplification, a point the Examiner does not dispute Godbout teaches. Using the teachings of Godbout regarding genes co-amplified based on selective advantages against Applicants' asserted utility is improper because selective advantage to cell survival is not the only mechanism by which genes impact cancer. Indeed, Godbout does not teach that gene amplification fails to correlate with mRNA

expression or polypeptide expression outside of the context of a gene conferring selective advantage.

Moreover, Applicants respectfully submit that the aspect of Godbout based on co-amplified genes does not apply to PRO357 because amplification of PRO357 was confirmed by epicenter mapping. Specifically, Applicants confirmed that amplification of the closest known epicenter markers did not occur to a greater extent than that of PRO357. Applicants teach that this "strongly suggests that the DNAs tested are responsible for the amplification of the particular region on the respective chromosome." Paragraph 0750. Thus, based on this teaching of the specification, one of ordinary skill in the art would conclude that PRO357 is not a co-amplified gene but rather an amplified gene.

Nor does the final reference relied on by the Examiner establish that Applicants' assertion of utility is inadequate. Similar to Godbout, Li teaches that "genes that are concurrently amplified because of their location with respect to amplicons" generally do not show correlation between gene amplification and mRNA or polypeptide overexpression. Applicants respectfully disagree that Li is persuasive evidence in the context of the present invention. As discussed above, framework and epicenter mapping analyses were carried out for PRO357 to confirm that PRO357, and not some other gene, is responsible for the observed gene amplification. This coupled with the high rates of observed amplification (approximately 2 to 8 fold amplification in nearly 62% of all tissues tested) indicates that PRO357 gene amplification more likely than not correlates with overexpression of the PRO357 polypeptide.

Moreover, according to the Examiner, Li is relevant for teaching that "68.8% of the genes showing overrepresentation in the genome did not show elevated transcript levels." (Page 7 of the Office action mailed 1/22/08). However, Applicants respectfully point out that Li acknowledged that their results differed from those obtained by Hyman and Pollack (both of record), who found a substantially higher level of correlation between gene amplification and increased gene expression. Li notes that "[t]his discordance may reflect that methodologic differences between studies or biological

differences between breast cancer and lung adenocarcinoma." (Page 2629, col. 1). For instance, as explained in the Supplemental Information accompanying the Li article, genes were considered to be amplified if they had a copy number ratio of at least 1.40. In the case of PRO357, as discussed in previously filed responses and in the Goddard Declaration (of record), an appropriate threshold for considering gene amplification to be significant is a copy number of at least 2.0 (which is a higher threshold). PRO357 showed significant amplification of $2^{1.05}$ to $2^{3.51}$ -fold and thus fully meets the standard of Li. The results of Li do not conclusively disprove that a gene with a substantially higher level of gene amplification, such as PRO357, would be expected to show a corresponding increase in transcript expression.

There is no requirement that only direct evidence of utility is sufficient to establish utility. Instead, it is established law that indirect evidence that is reasonably indicative of utility is sufficient to fulfill the requirements of 35 U.S.C. § 101. *Nelson v. Bowler*, 626 F.2d 853, 856 (CCPA 1980). Indeed, so long as there is a reasonable correlation between the evidence and the asserted utility, direct evidence is not needed. Applicants have demonstrated a reasonable correlation based on the references cited and discussed herein. Even if the references relied on by the Examiner provide some evidence that the correlation between Applicants' evidence and the asserted utility is not exact (Applicants do not concede that the cited references do provide such evidence), Applicants evidence still is sufficient to satisfy the utility requirement. See *Fujikawa v. Wattanasin*, 93 F.3d 1559 (Fed. Cir. 1996) (stating that "a 'rigorous correlation' need not be shown in order to establish practical utility; 'reasonable correlation' suffices").

Maintained Rejection under 35 U.S.C. § 102:

Rejection of claims 27-34 under 35 U.S.C. § 102 as allegedly being anticipated by Botstein (WO 99/35170) is maintained. Specifically, the Office action sets forth new grounds of rejection under 35 U.S.C. §§ 101 and 112 and concludes that "the claims are not entitled to the benefit of the filing date of the earlier filed applications" because of these rejections. Page 3 of the Office action mailed 1/22/08.

Applicants respectfully disagree with this ground of rejection. Applicants have overcome the new rejections based on the evidence and arguments submitted herein and continue to rely on their prior arguments. Specifically, Applicants previously noted that an application for a patent based on the present invention was filed at least as early as December 22, 1998 (see US Provisional Application Serial No. 60/113,296), which is prior to the publication date of the cited reference. For the reasons discussed above, the gene amplification experiment discussed in both the present application and provisional application 60/113,296 satisfies the requirements of 35 U.S.C. §§ 101 and 112. As such, Applicants respectfully submit that rejection of claims 27-34 under 35 U.S.C. § 102(b) based on the Botstein reference (WO 99/3517, published 7/15/99) is improper and respectfully request that this ground of rejection be withdrawn.

New Rejection under 35 U.S.C. § 112, Second Paragraph

Claims 27, 30-31, and 33-34 are rejected under 35 U.S.C. § 112, second paragraph, as allegedly indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention. The Office action alleges "extracellular domain" as set forth in the claims is indefinite. Page 11 of the Office action mailed 1/22/08.

Applicants respectfully disagree. This rejection is flawed because it incorrectly requires the term "extracellular domain" be defined in the claims. That is not the standard for definiteness. Instead, the test is whether "those skilled in the art would understand what is claimed when the claim is read in light of the specification." *Orthokinetics, Inc. v. Safety Travel Chairs, Inc.*, 806 F.2d 1565, 1576 (Fed. Cir. 1986). The claims here cannot be indefinite because the specification explains what "extracellular domain" refers to:

The PRO polypeptide "extracellular domain" or "ECD" refers to a form of the PRO polypeptide which is essentially free of transmembrane and cytoplasmic domains. Ordinarily, a PRO polypeptide ECD will have less than 1% of such transmembrane and/or cytoplasmic domains and preferably, will have less than 0.5% of such domains. It will be understood that any transmembrane domains identified for the PRO polypeptides of the present invention are identified pursuant to criteria routinely employed in the art for identifying that type of hydrophobic domain. The exact boundaries of a transmembrane domain may vary but most likely by no more than about 5 amino acids at either end of the domain as initially identified herein.

Optionally, therefore, an extracellular domain of a PRO polypeptide may contain 5 or fewer amino acids on either side of the transmembrane domain/extracellular domain boundary as identified in the Examples or specification and such polypeptides, with or without the associated signal peptide, and nucleic acid encoding them are contemplated by the present invention.

See paragraph 178. Figure 26 provides further information to assist one of ordinary skill in the art in understanding the specification's explanation of "extracellular domain." For example, Figure 26 identifies the transmembrane domain as located from approximately amino acids 501-522 of the 598 amino acid sequence of PRO357. However, solely to expedite prosecution, and not for reasons related to patentability, Applicants have herein amended claim 27 by deleting language relating to "extracellular domain" and cancelled claims 30-31. These amendments are made without disclaimer or prejudice to pursuing such claims in continuing applications.

The Office action also alleges recitation of "signal peptide" is indefinite. However, again the specification provides guidance. Specifically at Figure 26, the specification identifies the signal sequence as occurring from amino acids 1-23.

Only when a claim is "insolubly ambiguous" is it indefinite. *Metabolite Labs., Inc. v. Lab. Corp. of Am. Holdings.*, 370 F.3d 1354, 1366 (Fed. Cir. 2004). Claims 27, 30-31, and 33-34 are not rendered "insolubly ambiguous" by recitation of "signal sequence" since the specification provides guidance. Applicants respectfully submit this ground of rejection is overcome for claims 27, 30-31, and 33-34 and respectfully request it be withdrawn.

Rejection under 35 U.S.C. § 112, first paragraph:

Written Description

Claims 27, 30-31, and 33-34 are rejected under 35 U.S.C. § 112, first paragraph for allegedly failing to comply with the written description requirement. Specifically, the office action alleges that "the scope of the claims includes a genus of isolated polypeptides that comprise the amino acid sequence of some undisclosed 'extracellular domain' of SEQ ID NO:69 and chimeric polypeptides comprising such."

Pages 12-13 of the Office action mailed 1/22/08.

Application No. 09/943,780
Reply to Office Action of 1/22/2008
Request for Continued Examination Dated 4/22/2008

Solely to expedite prosecution, and not for reasons related to patentability, Applicants have herein amended claim 27 by deleting language relating to "extracellular domain" and cancelled claims 30-31. These amendments are made without disclaimer or prejudice to pursuing such claims in continuing applications.

The Examiner kindly acknowledges that the isolated polypeptides comprising the amino acid sequence of SEQ ID NO:69, optionally lacking its associated signal peptide, meets the written description requirement. Page 14 of the Office action mailed 1/22/08. Applicants respectfully submit that this ground of rejection is overcome and respectfully request it be withdrawn.

CONCLUSION

Applicants respectfully request the Examiner grant allowance of claims 27-29 and 32-34. The Examiner is invited to contact the undersigned attorney for the Applicant via telephone if such communication would expedite this application.

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PRAD-1/Cyclin D1 Gene Amplification Correlates with Messenger RNA Overexpression and Tumor Progression in Human Laryngeal Carcinomas¹

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ABSTRACT

PRAD-1 is a putative oncogene localized on chromosome 11q13 which encodes cyclin D1, a novel cyclin involved in cell cycle regulation. Amplification of this gene has recently been reported in several human tumors including breast and head and neck carcinomas. In this study we have analyzed the presence of *PRAD-1/cyclin D1* gene amplification and mRNA overexpression in a series of 46 matched normal mucosas and squamous cell carcinomas of the larynx. *PRAD-1/cyclin D1* was found to be amplified 2- to 12-fold in 17 carcinomas (37%). DNA amplification correlated with advanced local invasion ($P = 0.0015$), presence of lymph node metastases ($P = 0.0078$), and stage IV of the tumors ($P = 0.0021$). mRNA overexpression was found in 15 of the 43 (35%) cases examined and it was also significantly associated with advanced local invasion ($P = 0.0025$) and stage IV carcinomas ($P = 0.0032$). A significant association was observed between gene amplification and mRNA overexpression ($P < 0.0001$) with only 3 discordant cases (2 amplifications with no overexpression and 1 overexpressed carcinoma with no gene amplification). Furthermore, the degree of DNA amplification correlated with the levels of mRNA expression ($r = 0.6$; $P = 0.024$). These findings suggest that the *PRAD-1/cyclin D1* gene may be an important target of 11q13 amplifications in laryngeal carcinomas and the activation of this gene may be involved in the progression of these tumors. Its association with advanced-stage tumors indicates that *PRAD-1/cyclin D1* gene amplification and overexpression may be of prognostic significance.

INTRODUCTION

SCC⁷ of the larynx is an aggressive malignant neoplasm relatively frequent in certain countries (1). Epidemiological studies have identified personal habits (tobacco and alcohol), environmental, and viral, as major factors in the pathogenesis of laryngeal and other head and neck carcinomas (1). Malignant conversion of laryngeal mucosa is a progressive process in which several premalignant lesions have been morphologically recognized (2). The molecular mechanisms implicated in the malignant transformation and progression of these neoplasms are not well known.

Cytogenetic analysis of head and neck SCC have identified frequent chromosome abnormalities at 11q13, including clonal rearrangements and presence of homogeneously stained regions (3, 4). Several molecular studies have shown amplification of genes located

on this band (*int-2*, *hst-1*, and *bcl-1* locus) in 30–50% of the SCC of head and neck (5–8). Similarly, amplification of genes located on the 11q13 region may be involved in the development of other solid tumors such as lung (9), esophageal (10, 11), breast (5, 12–14), urinary bladder (15), and hepatocellular carcinomas (16). However, in spite of their amplifications, no RNA expression of *int-2*, *hst-1*, or *bcl-1* locus have been consistently demonstrated in fresh tumors or cell lines, suggesting that none of them are significant targets of the 11q13 amplification (6, 17).

PRAD-1 is a gene localized on chromosome 11q13 which was originally isolated as a gene clonally rearranged and overexpressed in parathyroid adenomas (18). This gene also seems to be the *bcl-1*-related gene activated by the t(11;14) translocation in some B-cell lymphomas/leukemias (19). *PRAD-1* encodes a novel cyclin called cyclin D1 which may play an important role in the control of the cell cycle at the G₁-S transition, probably by interacting with the retinoblastoma gene product (20, 21). Recently, transfection studies have demonstrated that *PRAD-1/cyclin D1* may function as a cooperating oncogene in the malignant transformation of cells (22). *PRAD-1/cyclin D1* gene may also be a target of 11q13 amplifications in solid tumors. Amplification and overexpression of this gene have been observed in breast and squamous cell carcinomas (17, 23–26). However, overexpression in squamous cell carcinomas has been mainly investigated in tumor cell lines (17, 23–25) and, therefore, the role of this gene in the progression of these tumors is unknown.

In this study we have analyzed *PRAD-1/cyclin D1* gene amplification and mRNA expression in a large series of human laryngeal carcinomas, and the results were correlated with the clinical and pathological characteristics of the patients.

MATERIALS AND METHODS

Patients and Tissues. A total of 46 human SCC of the larynx were obtained from 46 male patients (median age, 61 years, range, 39 to 92) who underwent surgery in our hospital from January 1992 to June 1993. Samples from tumor areas and nonneoplastic adjacent mucosas were snap-frozen in isopentane precooled in liquid nitrogen and stored at –80°C until studied. The remaining specimen was fixed in formalin and routinely processed.

The anatomical location of the tumors was glottic in 12 (26%), supraglottic in 16 (35%), pyriform sinus in 2 (4%), and transglottic in 16 (35%) (Table 1). Tumors were classified as keratinizing in 28 cases (61%) and nonkeratinizing in 18 (39%). The grade of differentiation was established according to Broders' criteria. Grades 1 and 2 were considered as low-grade carcinomas (15 cases, 32%) and Grades 3 and 4 as high-grade carcinomas (31 cases, 68%). Staging of tumors was established according to the American Joint Committee on Cancer (1992) (27). Thus, 2 patients had stage I carcinoma (4%), 3 had stage II (6%), 20 had stage III (44%), and 21 had stage IV (46%).

DNA Extraction and Southern Blot Analysis. High-molecular-weight DNA was isolated from frozen tissue of 46 carcinomas and matched normal mucosas. Cryostat sections of these cases were previously examined in order to determine the proportion of normal and neoplastic tissue present in each sample. Tumor samples were selected only if more than 75% of the section had carcinoma. DNA was extracted by conventional methods (28), and 10 µg were

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⁷ The abbreviations used are: SCC, squamous cell carcinoma; SSC, standard saline citrate (1 X SSC = 0.15 M NaCl, 0.015 M sodium citrate; pH 7.2); SDS, sodium dodecyl sulfate; RR, risk ratio.

digested with *Eco*RI. DNA from selected cases was also digested with *Bgl*II and *Hind*III. The samples were separated on 0.8% agarose gels and transferred to Hybond-N membranes (Amersham Int., Amersham, United Kingdom). The membranes were prehybridized with 50% formamide, 5X SSC, 5X Denhardt's, 500 µg/ml denatured salmon sperm DNA, at 42°C for 6 h, and hybridized with 50% formamide, 5X SSC, 1X Denhardt's, 100 µg/ml salmon sperm DNA, 10% dextran sulfate, and 10⁶ cpm/ml of ³²P-labeled PRAD-1 probe for 24 h. After hybridization, membranes were washed with 2X SSC, 0.1% SDS at room temperature for 30 min followed by 2X SSC, 0.1% SDS at 60°C for 30 min, and 0.1X SSC, 0.1% SDS at 60°C for 1 h. The filters were then autoradiographed by using intensifying screens at -70°C for 24–72 h. After being stripped free of the PRAD-1 probe, the same blots were hybridized with a ³²P-labeled β-actin probe to normalize against possible variations in loading or transfer of DNA.

The autoradiograms were analyzed by using a UVP-5000 video densitometer (UVP, San Gabriel, CA). Intensities of *PRAD-1/cyclin D1* bands were normalized to the β-actin control bands. The degree of amplification was calculated from these normalized values. Amplification was considered when the signal of the tumor band was ≥2-fold the value of its matched normal mucosa.

RNA Extraction and Northern Blot Analysis. Total RNA was isolated from frozen tissue of 43 carcinomas and matched normal mucosa by guanidine isothiocyanate extraction and cesium chloride gradient centrifugation (28, 29). Genomic DNA had been previously obtained in all cases. Eight µg of RNA from each sample were electrophoresed on a 1.2% agarose formaldehyde gel and transferred to Hybond-N membranes (Amersham). The PRAD1 complementary DNA probe was ³²P-labeled by random priming and hybridized to the blots at 45°C overnight. Washes after hybridization included a final step under stringent conditions in 0.1X SSC, 0.1% SDS at 65°C for 30 min. After being stripped free of the PRAD-1 probe, the same blots were hybridized with a ³²P-labeled 28S rRNA probe to normalize against possible variations in loading or transfer of RNA.

Hybridization signals of different radioautographic exposures within the linear response range were quantified as previously described, and the values of each case were normalized to the respective 28S rRNA signal. Overexpression was considered when the signal of the tumor band was 2-fold the value of the matched normal mucosa.

Probes. Probes were radiolabeled with the use of a random primer DNA-labeling kit (Promega Corp., Madison, WI) with [³²P]dCTP. The PRAD-1 probe used was a 1.4-kilobase *Eco*RI fragment (API-4) of the pPL-8 partial

complementary DNA clone of *PRAD-1* gene (kindly provided by Dr. A. Arnold, Massachusetts General Hospital, Boston, MA) (18).

Statistical Analysis. χ^2 test and Fisher's exact test were used for comparison between qualitative or categorical data. RR was computed according to previously described methods (30). Linear correlation of DNA amplification against RNA overexpression was carried out by means of the Pearson product moment correlation. Data were analyzed with the BMDP statistical software package (BMDP Statistical Software, Inc.).

RESULTS

***PRAD-1/cyclin D1* Gene Amplification in Laryngeal SCC.** The PRAD-1 probe detected 3 *Eco*RI fragments of 4.0, 2.2, and 2.0, and 1 *Bgl*II fragment of 15 kilobases as previously described (25). *PRAD-1/cyclin D1* gene amplification was detected in 17 of the 46 cases analyzed (37%). The degree of amplification was heterogeneous from tumor to tumor with a 2- to 12-fold variation when compared with the signal of the respective normal mucosa. Amplifications were confirmed with other restriction enzymes (Fig. 1 and 2). No rearrangements were seen in any tumor sample.

The correlations between gene amplification and the clinical and pathological characteristics of the patients are summarized in Tables 1–4. *PRAD-1/cyclin D1* gene amplification was significantly associated with an advanced stage of the tumors (Table 2). Amplification was found in 13 of the 21 (62%) stage IV carcinomas while only in 4 of the 25 (16%) stage I–III carcinomas ($P = 0.0021$; RR = 2.4). Similarly, DNA amplification was detected in 61% (11 of 18) of the carcinomas with extensive local invasion (T_4 carcinomas) but only in 22% (6 of the 28) of the carcinomas with limited invasion (T_1 – T_3 tumors) ($P = 0.0015$; RR = 3.4) (Table 3). There was also a significant correlation between the *PRAD-1/cyclin D1* gene amplification and the presence of lymph node metastases ($P = 0.0078$; RR = 5.9) (Table 4). Nonkeratinizing carcinomas were more frequently amplified (50%) than keratinizing tumors (29%) (Table 1). This difference, however, was not statistically significant. No association was observed between amplification and the age of the patient, histological grade, or localization of the tumor (Table 1).

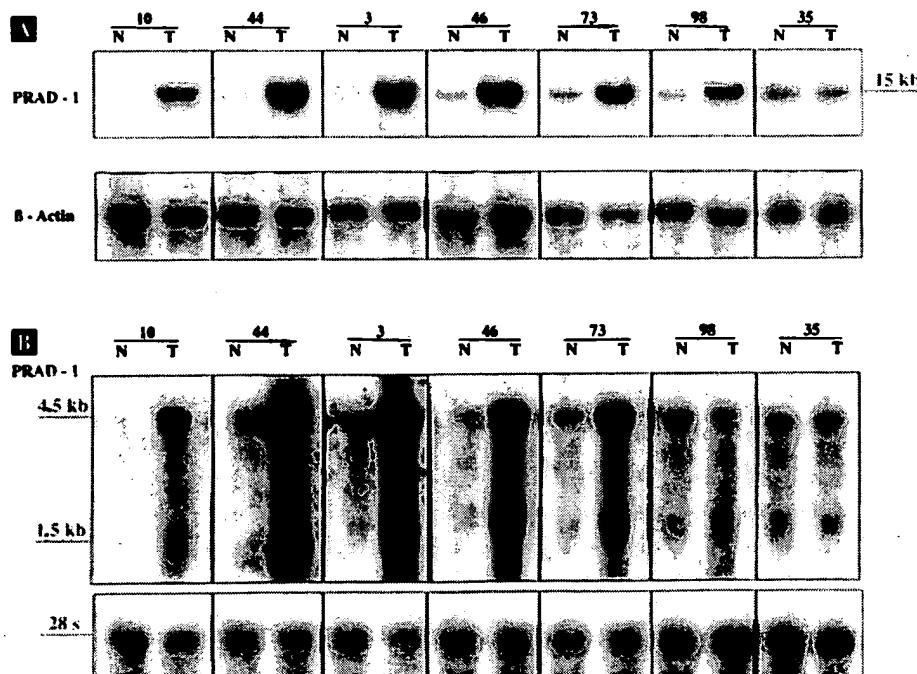


Fig. 1. *A*, Southern blot analysis of seven representative matched squamous cell carcinomas of the larynx (T) and normal mucosa (N) specimens. Numbers correspond to specific patients. Genomic DNA was digested with *Bgl*II and fractionated by electrophoresis in a 0.8% agarose gel. After transfer, the membranes were hybridized with PRAD-1 probe and subsequently with a β-actin probe for loading control. Tumors 10, 44, 3, 46, 73, and 98 show different degrees of *PRAD-1/cyclin D1* amplification, whereas tumor 35 was not amplified. *B*, Northern blot analysis of the same matched carcinomas (T) and normal mucosas (N) shown in *A*. Eight µg of total RNA were electrophoresed on a 1.2% agarose/formaldehyde gel and transferred to nylon membranes. The filters were hybridized to the PRAD-1 probe and subsequently to a 28S rRNA probe for loading control. Two transcripts of 4.5 and 1.5 kilobases were observed in all the samples. Tumors 10, 44, 3, 46, and 73 with DNA amplification also showed mRNA overexpression when compared to their respective normal mucosas. Tumor 98 was amplified but not overexpressed, and in case 35 neither amplification nor overexpression was detected.

Prad-1/Cyclin D1 mRNA Overexpression in Laryngeal SCC. In order to determine whether the *PRAD-1/cyclin D1* gene was transcribed in laryngeal carcinomas, total RNA was obtained from additional tissue available in 43 carcinomas and the respective nonneoplastic mucosa. On Northern blot analysis, the *PRAD-1* signal showed two transcripts of 4.5 and 1.5 kilobases. Low levels of these two transcripts were detectable in all normal samples. Fifteen carcinomas (35%) showed from 2- to 18-fold overexpression when compared to

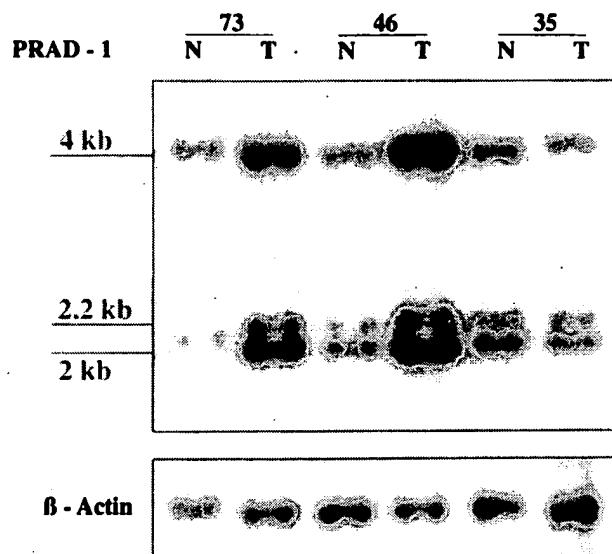


Fig. 2. Southern blot analysis of three carcinomas (T) and their respective normal mucosas (N). Genomic DNA was digested with *Eco*RI and fractionated by electrophoresis in a 0.8% agarose gel. After transfer, the membranes were hybridized with *PRAD-1* probe and subsequently with a β -actin probe for loading control. The identification of the 3 patients is the same as in Fig. 1. As observed with *Bgl*II, amplification was present in tumors 73 and 46 but not in case 35.

Table 1 *PRAD-1/cyclin D1* gene amplification/overexpression and pathological characteristics of the patients

	DNA amplification		mRNA overexpression	
	n	+(%)	n	+(%)
Age (median)	65	68	65	67
Keratinization				
Present	28	8 (29)	20 (71)	26 (23)
Absent	18	9 (50)	9 (50)	9 (53) ^a
Histological grade				
Low	15	5 (33)	10 (67)	14 (36)
High	31	12 (39)	19 (61)	29 (35)
Localization				
Supraglottic	16	5 (31)	11 (69)	13 (23)
Pyriform sinus	2	2 (100)	0 (0)	2 (100)
Glottic	12	3 (25)	9 (75)	12 (25)
Transglottic	16	7 (44)	9 (56)	16 (44)
Total	46	17 (37)	29 (63)	43 (35)

^a P = 0.047.

Table 2 Correlation between *PRAD-1* gene amplification/overexpression and tumor stage in laryngeal carcinomas

Stage	DNA		RNA	
	n	Amplification (%) ^a	n	Overexpression (%) ^b
I	2	0 (0)	2	0 (0)
II	3	1 (33)	3	0 (0)
III	20	3 (15)	18	3 (17)
IV	21	13 (62)	20	12 (60)
Total	46	17 (37)	43	15 (35)

^a Stage I-III versus stage IV (P = 0.0021).

^b Stage I-III versus stage IV (P = 0.0032).

Table 3 Correlation between *PRAD-1* gene amplification/overexpression and local invasion (T) in laryngeal carcinomas

	DNA		RNA	
	n	Amplification (%) ^a	n	Overexpression (%) ^b
T ₁	2	0 (0)	2	0 (0)
T ₂	4	1 (25)	4	0 (0)
T ₃	22	5 (23)	20	4 (20)
T ₄	18	11 (61)	17	11 (65)
Total	46	17 (37)	43	15 (35)

^a T₁₋₃ versus T₄ (P = 0.0015).

^b T₁₋₃ versus T₄ (P = 0.0025).

Table 4 Correlation between *PRAD-1* gene amplification/overexpression and lymph node metastases in laryngeal carcinomas

Metastasis	DNA		RNA	
	n	Amplification (%) ^a	n	Overexpression (%) ^b
Negative	37	10 (27)	35	10 (28)
Positive	9	7 (78)	8	5 (62)
Total	46	17 (37)	43	15 (35)

^a P = 0.0078.

^b P, not significant.

Table 5 Correlation between *PRAD-1/cyclin D1* gene amplification and mRNA overexpression in 43 laryngeal carcinomas (P < 0.0001)

mRNA overexpression	DNA amplification		
	n	Positive	Negative
Positive	15	14	1
Negative	28	2	26
Total	43	16	27

the signal of the matched mucosa. No anomalous transcripts were detected in any of the cases (Fig. 1).

The finding of mRNA overexpression in carcinomas correlated significantly with the presence of DNA amplification (Table 5) (P < 0.0001). Discordance between amplification and overexpression was observed in only three cases: two amplified tumors did not show mRNA overexpression whereas high levels of transcription were observed in one carcinoma in which Southern blot analysis did not detect gene amplification. The relationship between the degree of gene amplification and the level of mRNA expression was analyzed in the 14 cases with amplification and overexpression (Fig. 3). Linear analysis showed a significant correlation between these two parameters with an r value of 0.6 (P = 0.024).

As in gene amplification, a significant correlation was found between *PRAD-1/cyclin D1* overexpression and clinical progression of the disease (Tables 2, 3, and 4). mRNA overexpression was observed in 12 of the 20 (60%) stage IV carcinomas, but in none of the 5 stage I/II tumors, and in only 3 of the 18 (17%) stage III cases (P = 0.0032, RR = 2.8). The local invasion of carcinomas with high mRNA levels was also significantly more advanced than in tumors with normal expression of this gene (P = 0.0025, RR = 3.3) (Table 3). Overexpressed carcinomas were more frequently associated with lymph node metastases (62%) than tumors which were not overexpressed (28%) (Table 4), although this difference did not reach statistical significance. Nonkeratinizing carcinomas were more frequently overexpressed (53%) than keratinizing tumors (23%) (P = 0.047; Table 1). No correlation was observed between overexpression and the age of the patients, histological grade, or localization of the tumors (Table 1).

DISCUSSION

Amplification of 11q13 genes has been observed in 10-50% of squamous cell carcinomas of the head and neck, lung, and esophagus,

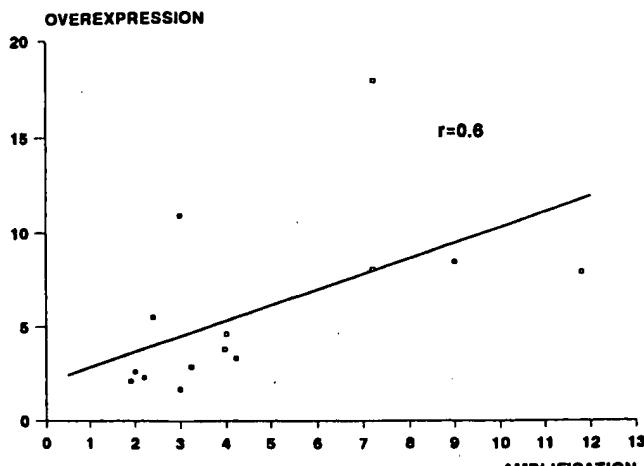


Fig. 3. Correlation between DNA amplification and mRNA overexpression in the 14 cases with *PRAD-1/cyclin D1* amplification and overexpression. The levels of mRNA overexpression are significantly related to the degree of DNA amplification ($r = 0.6$; $P = 0.024$).

indicating that genes located on this amplicon may play a role in the development and/or progression of these tumors (5-8, 10, 11). Occasional laryngeal carcinomas have been included in some of the head and neck series. However, no specific analysis has been performed in this group of carcinomas. In this study we have demonstrated that *PRAD-1/cyclin D1* is amplified in a relatively high number of SCC of the larynx and this amplification is associated with overexpression of the gene. Interestingly, amplification and overexpression were significantly more frequent in carcinomas with advanced local invasion, lymph node metastases, and, consequently, with an advanced tumor stage.

Amplification of the 11q13 region has been correlated with more advanced primary tumors and shortened survival in breast and esophageal carcinomas (11, 14, 31). There are some indications that amplification of 11q13 may also be associated with poorly differentiated and advanced head and neck carcinomas (6, 7). In these studies, however, the series examined was too small to show statistical significance. Two genes localized on this amplicon, *int-2* and *hst-1*, had been considered as candidates to be activated by this amplification. However, in contrast to the overexpression of *PRAD-1*, their levels of mRNA were consistently undetectable (6, 17). Our study confirms the relationship between 11q13 amplifications and poor prognosis parameters in SCC of the larynx. In addition, the consistent mRNA up-regulation of the *PRAD-1/cyclin D1* gene in our cases supports the idea that this gene plays an important role in the aggressive behavior of these lesions.

How overexpression of cyclin D1 may participate in the progression of these tumors is unknown. Recently, several studies have found that cyclin D1 overexpressing cells have abnormal proliferative characteristics with a shortened G_1 phase and less dependence on growth factors (20, 32). Cyclin D1 may also function as an oncogene cooperating with other oncogenes in cellular transformation (22). However, the tumorigenic and transforming properties of cyclin D1 seem to be less effective than the conventional oncogenes (20, 22). The overexpression of cyclin D1 in the late steps of the progression of human laryngeal carcinomas observed in our study suggests that this gene does not play a role in the initial transformation of squamous cells. Its up-regulation, however, may confer some growth advantage to cells already transformed by other mechanisms contributing to an increase in the aggressiveness of the tumor.

Association between *PRAD-1/cyclin D1* gene amplification and

overexpression has been observed in some cell lines (17, 23, 24). However, this correlation is more difficult to demonstrate in solid tumors because of tumor heterogeneity and the possible dilutional effect of nonneoplastic tissue present in the sample (23, 24). To minimize this problem only samples with more than 75% of tumor cells have been included in this study. The high correlation between overexpression and amplification observed in our study indicates that amplification is the main mechanism leading to *PRAD-1/cyclin D1* gene activation in SCC of the larynx. However, overexpression was observed without gene amplification in one case. Similarly, mRNA up-regulation with no gross genetic abnormalities has also been observed in some human breast carcinomas and solid tumor cell lines (17, 24). These findings suggest that alternative mechanisms of activating *PRAD-1/cyclin D1* gene such as translocations, mutations in gene regulatory regions, or stabilization of mRNA may also occur. *PRAD-1/cyclin D1* expression is activated by chromosome translocations and inversions in lymphoid neoplasms and parathyroid adenomas (18, 19). 11q13 translocations have been also described in some squamous cell carcinomas, suggesting that this mechanism may also activate this gene in this type of tumors (3, 20). Although no rearrangements were detected in the *PRAD-1/cyclin D1* gene in our case with the probe used in this study, we cannot rule out the presence of rearrangements involving more distant breakpoints as they occur in lymphoid neoplasms.

In our study two carcinomas showed *PRAD-1/cyclin D1* gene amplification without mRNA overexpression, suggesting that other genes in this amplicon may also be involved in the progression of some laryngeal carcinomas. *EMSI* and *EXP1* are recently identified genes in the 11q13 region which are frequently coamplified with *PRAD-1/cyclin D1* in breast and squamous cell carcinomas (24, 33, 34). In some cases, however, amplification of *EMSI* may occur with lower number of copies of the *PRAD-1/cyclin D1* gene (24, 26). Amplification of these genes is associated with their overexpression in some tumor cell lines and human carcinomas, suggesting that they may also be targets of this amplicon. Further studies are required to know whether these genes are also amplified and overexpressed in laryngeal carcinomas and to determine their possible cooperation in the progression of these tumors.

In conclusion, *PRAD-1/cyclin D1* gene is amplified in a high number of laryngeal carcinomas and this amplification is significantly associated with mRNA overexpression. Furthermore, *PRAD-1* amplification and overexpression correlate with advanced tumor invasion, lymph node metastasis, and advanced tumor stage. Our findings suggest that this gene is a relevant target of the 11q13 amplification in SCC of the larynx and may be involved in the progression of these tumors. The detection of cyclin D1 in SCC of the larynx may be a useful marker of prognostic significance.

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Short Communication

hTERT Gene Amplification and Increased mRNA Expression in Central Nervous System Embryonal Tumors

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High-level gains at 5p15, a chromosomal region including the human telomerase catalytic protein subunit (*hTERT*) gene, have been documented in several medulloblastomas. We therefore analyzed *hTERT* gene dosage in a group of medulloblastomas and other embryonal brain tumors using differential PCR. Amplification of the *hTERT* locus was detected in 15 of 36 (42%) tumors examined. To correlate gene amplification with message level, we used real-time quantitative PCR to measure *hTERT* mRNA in 50 embryonal brain tumors. *hTERT* mRNA was detected in all but one of these cases, and mRNA level correlated significantly with gene dosage ($r = 0.82$). Log-rank analysis of survival data revealed a trend toward poor clinical outcomes in patients with medulloblastomas containing high *hTERT* mRNA levels, but clinical follow-up was relatively short and the association was not statistically significant ($P = 0.078$). Comparative genomic hybridization was used to further analyze the tumor with the greatest *hTERT* gene dosage and mRNA level, a recurrent medulloepithelioma. *hTERT* was amplified in the recurrent tumor but not in the primary lesion, suggesting this locus can be involved in tumor progression. Our data indicate that *hTERT* gene amplification is relatively common in embryonal brain tumors, and that increased expression of *hTERT* mRNA may be associated with biologically aggressive tumor behavior. (Am J Pathol 2003; 162:1763–1769)

Brain tumors are the most common solid neoplasms that occur in childhood.¹ Among them, embryonal tumors are the most frequently encountered malignant lesions. In-

cluded in the current World Health Organization classification are medulloblastoma, supratentorial primitive neuroectodermal tumor (sPNET), atypical teratoid/rhabdoid tumor (AT/RT), and medulloepithelioma. The major molecular changes in central nervous system (CNS) embryonal tumors are only partially understood.² One gene commonly involved in carcinogenesis that has not yet been analyzed in a significant number of embryonal brain tumors is *hTERT*, which encodes the telomerase catalytic protein subunit.

Telomerase is an enzyme synthesizing the repetitive nucleotide sequence TTAGGG in telomeres. Human telomerase consists of an RNA subunit, (hTR), which is widely expressed, and a protein subunit, telomerase reverse transcriptase (hTERT), whose expression is more tightly regulated.^{3–5} The *hTERT* gene is located on chromosome 5 at 5p15.33; its expression is repressed in normal human somatic cells but is reactivated in most tumors (reviewed in⁶). In many neoplasms, increased telomerase activity is associated with poor clinical outcomes.^{6–11} While gene amplification has not generally been considered a common mechanism to increase telomerase activity in tumors, three recent reports have documented *hTERT* gene amplification in non-CNS primary tumors and tumor cell lines with concomitant increases in *hTERT* mRNA level.^{12–14}

Interestingly, high-level gains of chromosomal material in the 5p15 region have been detected in medulloblastomas, suggesting that the *hTERT* gene could be amplified in CNS embryonal tumors.^{15,16} Data on telomerase in these tumors is sparse. To our knowledge, *hTERT* gene dosage and mRNA levels have never been analyzed in

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Table 1. hTERT Molecular Analysis and Clinical Features in CNS Embryonal Tumors

Samples	Sex	Age	Status	Follow-up (months)	Histology	hTERT RT-PCR	hTERT/5qSTS
1	M	9	P	A 18	Classic MB	0.0	2.0
2	M	21	P	A 1	Classic MB	0.5	1.3
3	M	2	P	D 10	PNET	0.7	0.8
4	F	13	P	A 58	Classic MB	1.1	2.6
5	F	9	P	A 4	Nodular MB	1.2	NA
6	F	9	P	A 20	Anaplastic MB-F	1.3	0.6
7	F	2	P	A 9	Nodular MB	1.3	NA
8	F	4	P	A 8	Classic MB	1.8	6.0
9	M	5	P	A 5M	PNET	2.2	2.2
10	M	3	P	A 15	Nodular MB	2.4	NA
11	F	16	P	A 24	Anaplastic MB-F	2.7	1.2
12	M	23	P	A 75	Classic MB	3.5	1.9
13	F	<1	P	D 0	PNET	4.9	0.4
14	F	18	P	A 28	Anaplastic MB	5.4	0.4
15	M	11	R	D 27	Anaplastic MB	5.5	1.0
16	F	4	P	D 14	PNET	7.0	0.8
17	M	1	P	A 14	Nodular MB	8.5	NA
18	M	4	P	A 12	Nodular MB	10.0	NA
19	F	43	P	A 23	Anaplastic MB	13.8	1.7
20	M	4	P	A 9	Nodular MB	14.1	3.4
21	M	6	P	A 49	Anaplastic MB-F	15.5	0.3
22	M	11	R	A 73	Anaplastic MB	22.1	0.8
23	M	5	R	A 21	Medulloepithelioma	22.6	2.3
24	F	7	P	A 19	Anaplastic MB-F	26.9	NA
25	F	5	P	A 3	Anaplastic MB-F	28.6	0.5
26	M	1	P	D 0	PNET	30.3	2.1
27	M	2	P	D 17	Classic MB	31.0	NA
28	M	5	R	D 7	Anaplastic MB	31.8	3.1
29	M	6	P	A 22	Classic MB	36.5	NA
30	M	12	P	A 14	Classic MB	37.4	0.1
31	F	3	P	D 2	PNET	41.0	3.6
32	F	2	P	A 32	Anaplastic MB-F	48.1	0.4
33	F	16	P	A 28	Nodular MB	50.7	NA
34	M	6	P	A 25	Nodular MB	52.0	NA
35	M	9	P	D 9	Anaplastic MB	52.4	3.2
36	F	3	R	A 122	PNET	52.5	5.1
37	F	10	P	A 82	Classic MB	59.7	3.7
38	F	3	P	A 5	Classic MB	64.5	0.4
39	M	9	P	A 21	Nodular MB	64.8	NA
40	F	12	P	A 21	Anaplastic MB-F	65.2	NA
41	F	1	P	A 14	Anaplastic MB-F	88.9	NA
42	M	12	P	A 32	Medulloblastoma	125.1	1.2
43	M	39	R	A 58	Classic MB	125.2	6.0
44	F	4	P	D 3	PNET	129.5	2.5
45	M	26	P	A 21	Nodular MB	132.0	1.0
46	M	5	P	D 11	Anaplastic MB	133.0	1.6
47	F	55	P	A 9	Classic MB	144.1	NA
48	F	22	P	A 70	Pineoblastoma	152.3	3.2
49	M	15	R	D 116	Classic MB	154.0	3.7
50	F	1	R	D 31	Medulloepithelioma	603.6	17.6

* A, alive; D, deceased; F, focal; MB, medulloblastoma; P, primary; PNET, supratentorial primitive neuroectodermal tumor; R, recurrent.

medulloblastoma or other CNS embryonal neoplasms. In a recent review of telomerase in brain tumors, Falchetti and colleagues¹⁷ identified fewer than 10 CNS embryonal tumors from three studies in which telomerase enzymatic activity had been analyzed. We therefore used differential PCR and real-time RT-PCR to determine the relationship between *hTERT* gene copy number, *hTERT* mRNA expression, and clinical outcome in CNS embryonal tumors. We show that the *hTERT* gene is amplified in a significant number of cases, and that medulloblastoma patients with increased *hTERT* expression in their tumors have a trend toward worse clinical outcomes.

Materials and Methods

Clinical Samples

Tissue from 50 embryonal tumors resected between 1992 and 2002 at either the Johns Hopkins Hospital, Emory University Hospital, or L'hôpital Ste-Justine were used in these studies (Table 1). The cases included 15 anaplastic medulloblastomas, 13 classic medulloblastomas, 10 nodular medulloblastomas, 8 supratentorial PNET, 2 medulloepitheliomas, 1 medulloblastoma, and 1 pineoblastoma. The median age of patients was 7 years

(range, 6 months to 55 years) and 82% of the cases occurred in patients 18 years of age or less. The median follow-up for all patients was 19 months; the median follow-up in the medulloblastoma patients used for survival analysis was 20 months.

Molecular Analyses

DNA and total RNA were extracted from snap-frozen tumor tissues using TRIZOL Reagent (Invitrogen, Carlsbad, CA) per the manufacturer's instructions. RNA was then treated with DNase and further purified using the RNeasy Protocol (Qiagen, Valencia, CA). Quantitative RT-PCR was performed using the ABI Prism 7700 Sequence Detector (Applied Biosystems, Weiterstadt, Germany) with TaqMan One-Step RT-PCR Master Mix reagents (Applied Biosystems) according to the manufacturer's instructions. PCR primers used for the analysis of *hTERT* expression were *hTERT*-1912F (forward primer: 5'-TACGTCGTGGGAGGCCAGAAC-3') and *hTERT*-1978R (reverse primer: 5'-CCTTCACCCCTCGAG-GTGAGA-3'). The TaqMan probe *hTERT*-1933T (5'-TTC-CGCAGAGAAAAGAGGGCCGA-3') was labeled with 6-FAM and TAMRA. Amplicon length was 67 bp. Final concentration of primers was 0.5 μ mol/L; final concentration of the TaqMan probe was 0.2 μ mol/L. cDNA amount was standardized in each reaction using expression of β -actin according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). All samples were analyzed in triplicate. Serial dilutions of total RNA from the D425 medulloblastoma cell line (ATCC) were used to generate standard curves and the expression of *hTERT* in all samples was calculated in relation to this. Gene copy number of *hTERT* was determined using differential PCR of sequences from *hTERT* on 5p and control sequences on 5q (5qSTS). The *hTERT* product was 260 bp long. The forward and reverse primers were 5'-GTGACCGTGGTT-TCTGTGTG-3' and 5'-GGGCCTCAGAGAGCTGAGTA-3' respectively. The 5qSTS fragment was 199 bp in length; forward and reverse primers were 5'-TTGGTCCCCAG-TAACTTGATG-3' and 5'-TGTCCCCTAGGATCTTGCT-CAG-3'. PCR conditions are available on request. Bands were visualized and quantitated using a Fluor-S Multiimager (Bio-Rad, Hercules, CA). CGH was performed as previously described.¹⁸ The SYSTAT 9 program was used for Kaplan-Meier survival curves and Breslow-Ge-han log-rank analysis.

Results

The hTERT Gene is Amplified in 42% of CNS Embryonal Tumors

Amplification of the *hTERT* gene at 5p15.33 was detected using differential PCR following the methods of Waha and colleagues.¹⁹ The *hTERT*/5qSTS DNA ratios in the 36 tumors analyzed ranged from 0.1 to 17.6 (Table 1 and Figure 1). Experimental replicates were performed in selected cases and yielded similar results. Analysis of *hTERT*/5qSTS DNA ratios in 8 normal subjects produced

Case 45 15 11 50

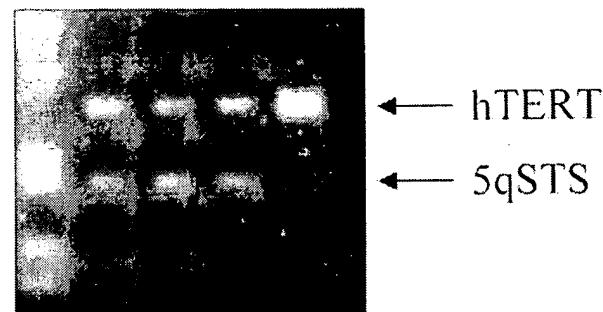


Figure 1. *hTERT* gene amplification detected by differential PCR. In cases 45, 15, and 11, *hTERT* and 5qSTS sequences are amplified in a roughly 1:1 ratio. In case 50, the *hTERT* product is 17-fold more abundant, consistent with gene amplification at the locus.

values ranging from 0.99 to 1.12, with a mean of 1.02. The *hTERT*/5qSTS DNA ratio threshold for amplification was therefore set at 2.17, which represents twice the mean ratio of the normal subjects plus three standard deviations, a standard established in earlier reports on this method.^{19,20} Using this cut-off, *hTERT* gene amplification was detected in 15 of 36 (42%) CNS embryonal tumors (Table 1). The *hTERT*/5qSTS ratios in amplified cases ranged from 2.2 to 17.6.

hTERT mRNA Expression Level and Clinical Factors

We used real-time RT-PCR to measure the level of *hTERT* in mRNA extracted from snap-frozen tumor tissue. *hTERT* mRNA was detected in 49 of 50 CNS embryonal tumors examined using this method. We divided these tumors into those with above-median *hTERT* expression, high *hTERT*, and below-median *hTERT* level, low *hTERT* (Table 1). *hTERT* mRNA expression levels did not distinguish between different types of embryonal tumors, eg, PNET, medullopithelioma, medulloblastoma, etc. The nodular, classic, and anaplastic medulloblastoma subtypes were also distributed roughly evenly with respect to *hTERT* level. However, as has been observed in other tumor types, high levels of *hTERT* were more common in patients who died from their disease. In the combined group of embryonal tumor patients, 9 of 25 (36%) with above-median *hTERT* died, compared to 4 of 25 (17%) with below-median *hTERT*. The survival difference was more pronounced within the diagnostically uniform group of medulloblastoma patients. Only one medulloblastoma patient with low *hTERT* died from his disease, compared to five deaths in the high *hTERT* medulloblastoma group. However, perhaps because of the relatively short follow-up time, log-rank analysis of Kaplan-Meier survival curves did not confirm significantly worse clinical outcomes in the high *hTERT* group of medulloblastoma patients ($P = 0.078$; Figure 2).

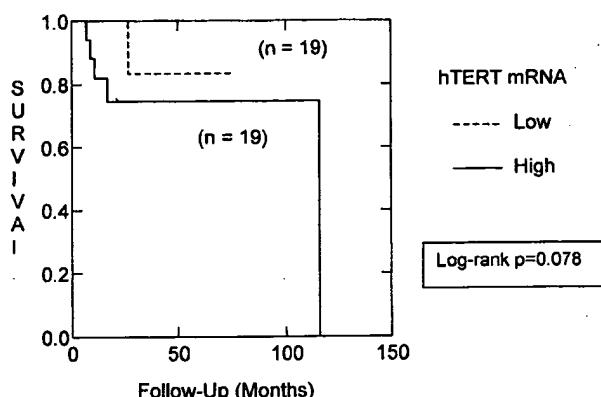


Figure 2. High hTERT expression is associated with poor clinical outcome. Log-rank analysis of Kaplan-Meier survival curves for patients with above or below median hTERT mRNA level reveals a trend toward better clinical outcomes for patients in the low hTERT group ($P = 0.078$).

Gene Amplification is Associated with Increased hTERT mRNA Expression

Gene amplification often results in increased expression from the amplified locus. We therefore correlated hTERT copy number with hTERT mRNA expression. Linear regression analysis showed a strong association between increasing hTERT DNA copy number and increasing hTERT mRNA level. When comparing hTERT/5qSTS ratio to hTERT mRNA, the correlation coefficient was 0.82, a statistically significant value for this number of samples ($P < 0.01$). However, the copy number of hTERT at the DNA level did not appear to correlate with survival, and log-rank analysis of survival in patients with tumors amplified or nonamplified at the hTERT locus revealed no trend toward worse outcomes in cases with hTERT amplification.

CGH Analysis of a Medulloepithelioma with hTERT Gene Amplification

We chose to further investigate the molecular alterations in case 50, which represented the most extreme example of hTERT gene amplification and had a very high hTERT message level as well. The hTERT/5qSTS DNA ratio and hTERT mRNA level were several-fold higher in this recurrent medulloepithelioma than in any other CNS embryonal tumor. The patient was a one-year-old white female diagnosed with a medulloepithelioma located in the left cerebellar hemisphere when she was 16 months old. Initial therapy consisted of cisplatin, oral VP-16, and craniospinal radiation followed by peripheral blood autologous stem cell transplant. Seventeen months after the first operation, the tumor recurred in the posterior fossa and was again resected. Despite additional therapy, the tumor continued to progress locally and the patient died three months later.

To confirm hTERT gene amplification, and to identify chromosomal changes occurring in medulloepithelioma, we analyzed DNA from both the original and the recurrent tumor. The chromosomal regions with DNA copy number

alterations in the primary and recurrent medulloepithelioma are illustrated in Figure 3A. There were 5 chromosomal alterations in the primary tumor and 17 in the recurrent lesion. The gains in the primary medulloepithelioma were localized to 3p13-22, 6p21.2-21.3, 14q24-qter, 15q15-25, and 20q; gains in the recurrent medulloepithelioma were at 1q32-qter, 3p14-22, 3q13-25, 5p14-pter, 7p15-q11, 9q34-qter, 10p, 12p, 14q, 15q24-qter, 17q21-qter, and 20q (Figure 3). There were two high-level gains in the recurrent tumor on chromosome 5p14-pter and 20q. Chromosome 5 profiles from the primary and recurrent tumor are shown in Figure 3B, where each line to the right or left of the central, dark band represents an incremental difference ratio of 0.25. Thus the difference ratio at the hTERT locus in the amplified recurrent tumor is over 2.0, a change equal to or higher than what we have previously observed in medulloblastomas highly amplified at the myc loci.¹⁵ There was no loss found in the primary medulloepithelioma. The losses in the recurrent medulloepithelioma involved 4p14-q28, 4q34-qter, 5q, 13q, and 18q12-qter.

Discussion

The hTERT gene is located on chromosome 5 at 5p15.33, a region that is sometimes gained in CNS embryonal tumors such as medulloblastoma.^{15,16} Recently, two groups demonstrated that the hTERT gene is amplified in several types of malignancy^{13,14}; however, to date, no information on hTERT amplification in solid tumors of the CNS has been published. We therefore analyzed hTERT gene dosage in a series of CNS embryonal tumors using comparative PCR, and identified hTERT gene amplification in 42% of the 36 samples examined. To our knowledge, this is the first analysis of hTERT gene copy number in CNS embryonal tumors, and one of only a few reports on hTERT gene amplification in any tumor type.

We identify a somewhat higher percentage of hTERT amplification in CNS embryonal tumors than previously reported for other tumor types. Using FISH, Zhang and colleagues¹³ found amplification of the hTERT gene in 31% of 26 tumor cell lines and 29% of 58 primary tumors examined, including lung tumors, cervical tumors, breast carcinoma, and neuroblastomas. The same group also described hTERT gene amplification in 24% of 88 cervical carcinomas, and an association between elevated hTERT protein expression and gene amplification.¹² A second group recently analyzed hTERT in lung cancer cell lines using several methods, documenting increased hTERT gene dosage in 6 of 20 samples. They also found a correlation between increased hTERT gene dosage, hTERT mRNA expression and telomerase activity.¹⁴

High hTERT expression or increased telomerase activity has correlated with clinical factors in a number of tumors, including Wilms' tumor, lymphoma, breast cancer, non-small-cell lung cancer, and urothelial carcinoma.¹¹⁻¹⁴ We therefore evaluated hTERT message levels in CNS embryonal tumors using quantitative RT-PCR, detecting mRNA in 49 of 50 tumors. In both the full group of CNS embryonal tumors and the clinically more homogeneous subset of individuals with medulloblastomas,

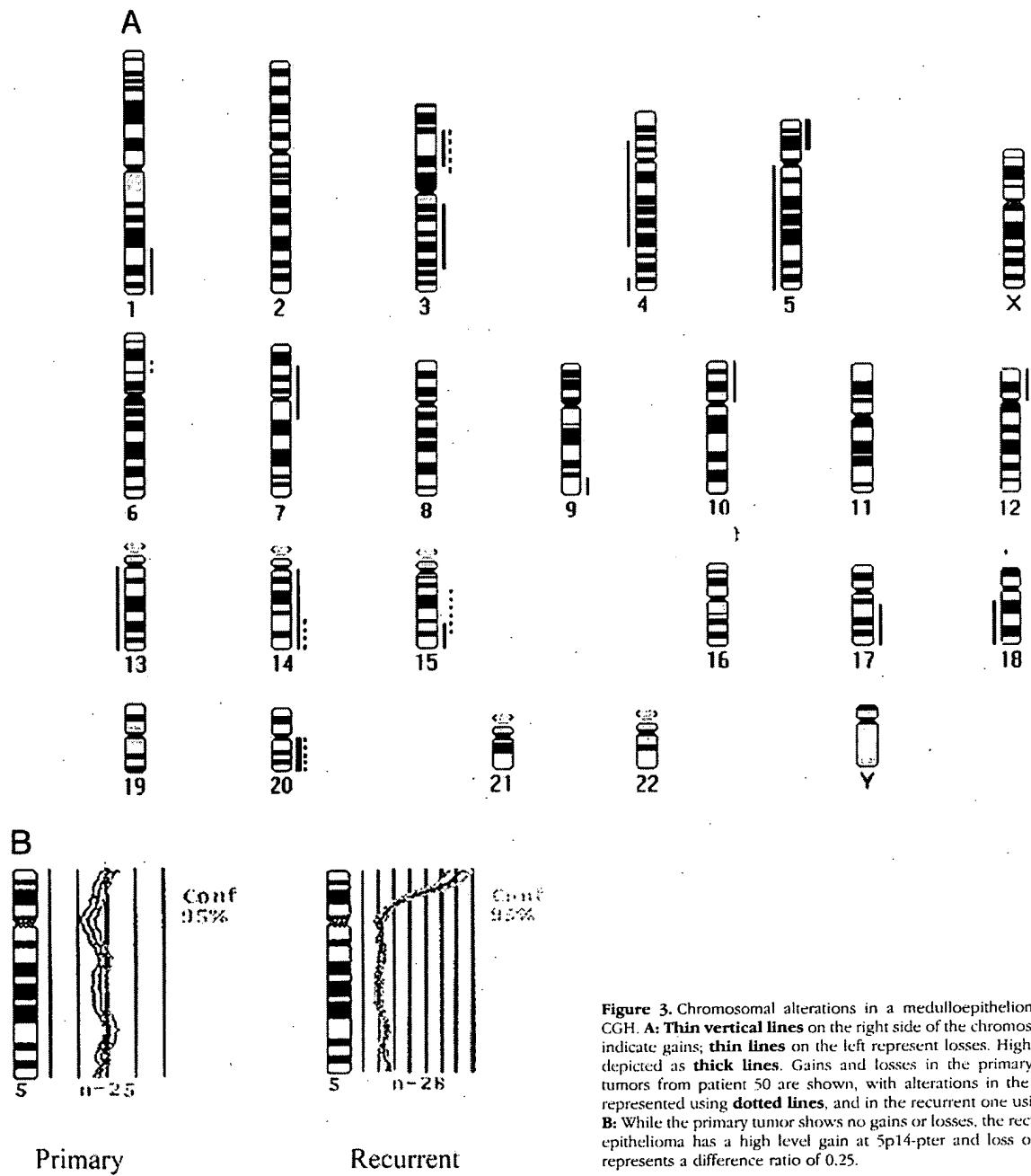


Figure 3. Chromosomal alterations in a medulloepithelioma detected by CGH. **A:** Thin vertical lines on the right side of the chromosome ideograms indicate gains; thin lines on the left represent losses. High-level gains are depicted as thick lines. Gains and losses in the primary and recurrent tumors from patient 50 are shown, with alterations in the primary lesion represented using dotted lines, and in the recurrent one using solid lines. **B:** While the primary tumor shows no gains or losses, the recurrent medulloepithelioma has a high level gain at 5p14-pter and loss of 5q. Each line represents a difference ratio of 0.25.

more than twice as many deaths were seen when tumors contained high levels of *hTERT* mRNA. However, log-rank analysis of survival curves did not confirm the significance of this difference ($P = 0.078$). Most of the patients involved in our study had their tumors removed within the last few years, and the follow-up available is therefore relatively short (median 20 months). This short follow-up period may have contributed to the lack of significance in our study, and additional investigations will be needed to confirm the prognostic power of *hTERT* expression in these CNS tumors.

It will also be important to correlate *hTERT* mRNA expression with telomerase enzymatic activity in CNS embryonal tumors, as the link between these remains uncertain. While

all of the telomerase positive brain tumors studied by Cabuy and de Ridder²¹ contained *hTERT* message, they failed to detect telomerase activity in some *hTERT*-expressing tumors. It has been suggested that the expression of non-functional *hTERT* splice variants can result in *hTERT* positive tumors that lack telomerase enzymatic activity.²² However, many authors report good correlations between *hTERT* message level and telomerase enzymatic activity.²³⁻²⁵

Intratumoral heterogeneity of *hTERT* expression and telomerase activity could have contributed to some of the differences reported in these earlier studies. Regional variations in *hTERT* expression and telomerase activity have been detected in some CNS gliomas.^{21,26} Similar heterogeneity at the level of *hTERT* DNA amplification or

hTERT expression could be present in the embryonal tumors we analyzed. We attempted to extend our studies of hTERT expression to the protein level using immunohistochemistry, which would also enable localization of expression to specific tumor regions. However, while the highly amplified medulloblastoma was diffusely immunopositive for hTERT, in the other 13 cases we found no correlation between DNA, RNA and protein levels (data not shown).

Gene amplification in tumors often results in increased mRNA expression from the amplified locus. In CNS embryonal tumors, we found a significant positive correlation between increasing gene dosage and hTERT message level. In some individual cases gene amplification was not associated with elevated message level, a finding similar to that observed for other oncogenes. For example, Grotzer and colleagues²⁷ recently found that c-myc gene amplification was only associated with high mRNA levels in a subset of medulloblastomas. Similarly, we have identified medulloblastomas amplified at the N-myc locus, but with low levels of N-myc mRNA (unpublished results). In 20 CNS embryonal tumors we detected hTERT mRNA, but no amplification was found at the locus by differential PCR. Presumably, other transcriptional regulators direct hTERT expression in these tumors. c-Myc,²⁸⁻³⁰ Sp1,³¹ and estrogen³² have all been implicated as hTERT transcriptional regulators (reviewed in³³), and could play a role in the diverse hTERT message levels we detected. The large number of embryonal tumors we found with unamplified *hTERT* and low-level hTERT mRNA expression also suggests that only modest telomerase expression levels may be required for formation of these neoplasms. Indeed, it is possible that telomerase-independent mechanisms of transformation are operative in PNET, as has been suggested for CNS gliomas.³⁴

We examined chromosomal alterations in a recurrent medulloblastoma with very high hTERT levels using CGH. High-level DNA gains were detected at 5p15, confirming the *hTERT* amplification identified using differential PCR. Interestingly, CGH analysis of the primary lesion from this patient detected no gains of 5p, indicating that *hTERT* amplification can first occur during tumor progression. However, most of the embryonal brain tumors with *hTERT* amplification in our study were not recurrent lesions, suggesting that as is the case in gliomas these alterations can occur early in tumorigenesis as well.³⁴ The overall number of chromosomal abnormalities was much higher in the recurrent medulloblastoma than in the primary one, suggesting molecular progression can occur in these rare embryonal neoplasms. A similar accumulation of chromosomal changes has been observed in recurrence or metastasis of medulloblastomas.^{15,35} Thus our CGH analysis of this medulloblastoma adds to the growing body of evidence suggesting CNS embryonal tumors can progress both microscopically and molecularly.

In conclusion, we have shown that the *hTERT* oncogene is amplified in a significant proportion of medulloblastomas and other CNS embryonal neoplasms. This gene amplification correlates with increased expression of hTERT mRNA, and implicates, for the first time, changes at the *hTERT* locus

in the evolution of primitive neuroepithelial tumors of the CNS. Finally, our correlation of hTERT expression with survival suggests hTERT message level could be a useful molecular prognostic marker.

Acknowledgments

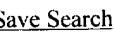
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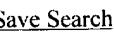
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hTERT gene dosage correlates with telomerase activity in human lung cancer cell lines.

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Maintenance of telomeres, most often by telomerase, is a necessary prerequisite for immortality of eukaryotic cells. To better understand the mechanisms of telomerase up-regulation during tumorigenesis, we analysed the gene dosage of hTERT on chromosome 5p15, a region known to be overrepresented in a variety of malignancies, in 20 lung cancer cell lines by Southern blotting, fluorescence in-situ hybridization, and comparative genomic hybridization. We found a significant correlation between hTERT gene dosage, hTERT mRNA expression and telomerase activity. Imbalances of chromosome 5p may exert functionally relevant hTERT gene dosage effects in human lung cancer.

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Gene amplification and expression of the DNA repair enzyme, N-methylpurine-DNA glycosylase (MPG) in HPV-infected cervical neoplasias.

Sohn TJ, Kim NK, An HJ, Ko JJ, Hahn TR, Oh D, Lee SG, Roy R, Cha KY, Oh YK.

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BACKGROUND: Lethal and mutagenic damages of DNA is caused by a variety of agents including viruses. It is known that HPV is one of the major causes of cervical carcinogenesis and that cells eliminate DNA lesions with DNA repair enzymes. However, the role of N-methylpurine-DNA glycosylase (MPG) is not known in the development of cervical cancer.

MATERIALS AND METHODS: Multiplex polymerase chain reaction (PCR) was used for the detection and typing of HPV in the biopsy. Gene amplification of MPG was measured by a PCR-based assay. The mRNA levels of MPG were determined by reverse transcription-PCR using hypoxanthine-guanine phosphoribosyl transferase as the reference gene. An immunohistochemical technique was used to examine the distribution of MPG in the tissues. **RESULTS:** Of 68 Korean cervical neoplasia patients, 86.8% showed HPV infection. High-risk HPV 16/18 were the most prevalent but positive only in 47.3% of the invasive cancer patients. Gene amplification of MPG was significantly increased in high-risk HPV-infected tissues as compared to low-risk HPV-infected and normal tissues ($p < 0.05$). The mRNA levels of MPG were higher in HPV-infected invasive carcinoma than normal cervical tissues. Immunohistochemical staining revealed that the intracellular expression and distribution (localization) of MPG altered in the cervical neoplasia. Interestingly, MPG expression in CIN III and invasive carcinoma (IC) was much higher than normal and CIN I. Granular positivity of MPG was notable in the perinuclear regions of the cytoplasm in HPV-infected invasive cancer. **CONCLUSION:** This is the first report on MPG expression in cervical neoplasia. Our results indicate that the gene amplification and expression of MPG were increased in high-risk HPV-infected cervical neoplasias and the intracellular distribution of MPG protein was altered, suggesting a role of MPG in carcinogenesis.

PMID: 11724299

Amplification and overexpression of PRUNE in human sarcomas and breast carcinomas-a possible mechanism for altering the nm23-H1 activity.

Forus A, D'Angelo A, Henriksen J, Merla G, Maelandsmo GM, Flørenes VA, Olivieri S, Bierkehagen B, Meza-Zepeda LA, del Vecchio Blanco F, Müller C, Sanvitto F, Kononen J, Nestland JM, Fodstad Ø, Reymond A, Kallionиемi OP, Arrigoni G, Ballabio A, Myklebost O, Zollo M.

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PRUNE, the human homologue of the *Drosophila* gene, is located in 1q21.3, a region highly amplified in human sarcomas, malignant tumours of mesenchymal origin. Prune protein interacts with the metastasis suppressor nm23-H1, but shows impaired affinity towards the nm23-H1 S120G mutant associated with advanced neuroblastoma. Based on these observations, we previously suggested that prune may act as a negative regulator of nm23-H1 activity. We found amplification of PRUNE in aggressive sarcoma subtypes, such as leiomyosarcomas and malignant fibrous histiocytomas (MFH) as well as in the less malignant liposarcomas. PRUNE amplification was generally accompanied by high mRNA and moderate to high protein levels. The sarcoma samples expressed nm23-H1 mostly at low or moderate levels, whereas mRNA and protein levels were moderate to high in breast carcinomas. For the more aggressive sarcoma subtypes, 9/13 patients with PRUNE amplification developed metastases. A similar situation was observed in all breast carcinomas with amplification of PRUNE. Infection of NIH3T3 cells with a PRUNE recombinant retrovirus increased cell proliferation. Possibly, amplification and overexpression of PRUNE has the same effect in the tumours. We suggest that amplification and overexpression of PRUNE could be a mechanism for inhibition of nm23-H1 activity that affect the development or progression of these tumours.

PMID: 11687967 [PubMed - indexed for MEDLINE]

Her-2/neu gene amplification, elevated mRNA expression, and protein overexpression in the metaplasia-dysplasia-adenocarcinoma sequence of Barrett's esophagus

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SUMMARY: The importance of alterations of the Her-2/neu oncogene in the tumorigenesis of Barrett's adenocarcinoma (BCA) is discussed controversially. In the present study, we evaluated for the first time the Her-2/neu status in the metaplasia-dysplasia-adenocarcinoma sequence of BCA simultaneously at the DNA, mRNA, and protein level using resection specimens of 25 patients. The locus-specific Her-2/neu gene status was quantified by performing fluorescence *in situ* hybridization, and information about the ploidy status of chromosome 17 was obtained. Tissue sections from the same areas were used for quantitative RT-PCR (TaqMan RT-PCR) of laser-microdissected tumor cells and for immunohistochemistry to quantify Her-2/neu mRNA and oncoprotein expression. Her-2/neu gene amplification was observed in 35% of BCA, and all of these samples showed strong overexpression of both mRNA and oncoprotein. A polysomy 17 without Her-2/neu gene amplification was observed in 52% of BCA, showing a normal or moderately elevated mRNA expression and no or weak immunopositivity. From 13 areas of high-grade dysplasia (HGD) we found four to be amplified for the Her-2/neu locus, whereas five showed a polysomy 17. All four samples of HGD areas with Her-2/neu gene amplification displayed mRNA and strong oncoprotein overexpression; however, lower mRNA levels were seen than in the amplified BCA areas. None of the samples with low-grade dysplasia (LGD) showed a locus-specific Her-2/neu amplification, but polysomy 17 was present in four of eight cases. No changes were detected in BCA-associated intestinal metaplasia and squamous epithelium. In summary, only a locus-specific Her-2/neu gene amplification was associated with strong mRNA overexpression and strong membranous Her-2/neu immunostaining in BCA and HGD. A chromosome 17 polysomy, as found in the majority of BCA, led to no or weak mRNA overexpression and no or weak immunopositivity. In the metaplasia-dysplasia-adenocarcinoma sequence, a chromosome 17

polysomy without Her-2/neu gene amplification was already present in LGD. This may be a result of an early polyploidization, preceding the later genetic events, such as Her-2/neu gene amplification in HGD and BCA.

PMID: 11406641

An immunohistochemical and *in situ* hybridization study of c-myc and c-erbB-2 expression in primary human breast carcinomas.

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In previous studies of the expression and organization of proto-oncogenes in human breast a significant correlation has been found between amplification of c-myc and c-erbB-2 genes in carcinomas and poor short-term prognosis. Gene expression was estimated by analysis of total RNA from tissues, and similarly assessment of gene organization relied upon extraction of DNA from tissues. The present study has compared the expression of c-myc and c-erbB-2 mRNA as determined by *in situ* hybridization, and c-myc and c-erbB-2 protein expression detected by immunohistochemistry in a group of carcinomas for which there was knowledge of genomic organization and/or expression. Formalin-fixed, paraffin-embedded tissues of 38 carcinomas were assessed for the presence of c-myc protein, and 13 of these were examined for c-myc mRNA by *in situ* hybridization. Similarly processed tissue from 14 tumours was tested for c-erbB-2 protein using the antiserum 21N and ten of these carcinomas studied for c-erbB-2 mRNA localization. There was a good correlation between gene amplification, the presence of c-erbB-2 protein and mRNA: both the latter were detected in six of the seven carcinomas with an amplification but in none without. For some carcinomas there was a good correlation between c-myc protein and mRNA levels. Three carcinomas with gene amplification had a lower percentage of cells with detectable protein than showed hybridization for mRNA. Other carcinomas had a lower level of mRNA expression than protein. Neither approach could predict which carcinomas had amplification of the c-myc gene.(ABSTRACT TRUNCATED AT 250 WORDS)

PMID: 2569035

Correlation of amplification and overexpression of the c-myc oncogene in high-grade breast cancer: FISH, in situ hybridisation and immunohistochemical analyses.

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In this study, we analysed gene amplification, RNA expression and protein expression of the c-myc gene on archival tissue specimens of high-grade human breast cancer, using fluorescent *in situ* hybridisation (FISH), nonradioactive *in situ* hybridisation and immunohistochemistry. The specific question that we addressed was whether expression of c-Myc mRNA and protein were correlated with its gene copy amplification, as determined by FISH. Although c-Myc is one of the most commonly amplified oncogenes in human breast cancer, few studies have utilised *in situ* approaches to directly analyse the gene copy amplification, RNA transcription and protein expression on human breast tumour tissue sections. We now report that by using the sensitive FISH technique, a high proportion (70%) of high-grade breast carcinoma were amplified for the c-myc gene, irrespective of status of the oestrogen receptor. However, the level of amplification was low, ranging between one and four copies of gene gains, and the majority (84%) of the cases with this gene amplification gained only one to two copies. Approximately 92% of the cases were positive for c-myc RNA transcription, and essentially all demonstrated c-myc protein expression. In fact, a wide range of expression levels were detected. Statistically significant correlations were identified among the gene amplification indices, the RNA expression scores and protein expression scores. c-myc gene amplification, as detected by FISH, was significantly associated with expression of its mRNA, as measured by the intensity of *in situ* hybridisation in invasive cells ($P=0.0067$), and by the percentage of invasive cells positive for mRNA expression ($P=0.0006$). c-myc gene amplification was also correlated with the percentage of tumour cells which expressed high levels of its protein, as detected by immunohistochemistry in invasive cells ($P=0.0016$). Thus, although multiple mechanisms are known to regulate

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MYCN Amplification and Expression

Cytogenetic analyses of neuroblastoma frequently show extrachromosomal double minutes or chromosomally integrated homogeneously staining regions, both of which are manifestations of gene amplification. The region amplified is virtually always derived from the distal short arm of chromosome 2, and contains the protooncogene *MYCN*.⁴¹ Brodeur, Seeger, and colleagues originally demonstrated that *MYCN* amplification occurs in approximately 25% of primary neuroblastomas from untreated patients, and amplification is associated with advanced stages of disease, rapid tumor progression, and a poor prognosis.^{42, 43} Amplification is found in 30% to 40% of patients with advanced disease and in only 5% to 10% of patients with low stages of disease and stage 4S.^{2, 44-}⁴⁶ *MYCN* amplification is almost always present at the time of diagnosis, if it is going to occur,⁴⁷ so it appears to be an intrinsic biologic property of a subset of very aggressive tumors. Interestingly, targeted overexpression of a *MYCN* transgene to the murine peripheral neural crest causes neuroblastic tumors with high penetrance and with remarkably similar phenotype as that seen in humans.⁴⁸

In general, there is a correlation between *MYCN* copy number and expression at the messenger ribonucleic acid (mRNA) and protein levels.⁴⁹ Thus, *MYCN* overexpression in the context of amplification consistently identifies a subset of neuroblastomas with highly malignant behavior. However, it is controversial whether or not overexpression of *MYCN* has prognostic significance in nonamplified

tumors.^{49, 50} Some neuroblastoma cell lines express high levels of *MYCN* mRNA or protein without gene amplification.⁴⁹⁻⁵¹ This may be a result of alterations in normal protein degradative pathways, rather than loss of *MYCN* transcriptional autoregulation.^{52, 53} In addition, some studies have suggested that MycN expression correlates inversely with survival probability,⁵⁴ whereas others have found no such correlation.⁵⁵ Further studies in a larger cohort of consistently treated patients will be necessary to determine if quantitative assessment of MycN expression in tumors lacking *MYCN* amplification provides additional prognostic information.

CENP-F gene amplification and overexpression in head and neck squamous cell carcinomas.

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BACKGROUND: Antibodies against cancer-related genes have been detected in human cancers including head and neck cancers. High titers of c-Myc autoantibodies have been linked to gene amplification and tumor progression. Centromere protein-F (CENP-F) autoantibodies have been detected in patients with various cancers, suggesting similar gene alteration.

METHODS: CENP-F and c-MYC amplification was assessed in 72 head and neck squamous cell carcinoma (HNSCC) patients. Tumor and matched mucosa from 22 patients were analyzed for CENP-F mRNA levels by RT-PCR. **RESULTS:** The larynx was the site most altered by amplification of either gene. CENP-F and c-MYC were amplified in 11% and 17% of the tumors, respectively. Coamplification was found in 7% of the tumors, most of which showed regional node involvement. CENP-F mRNA was overexpressed in 36% of tumors, and 23% of paired mucosa. **CONCLUSION:** Our results provide the first evidence that CENP-F gene is amplified and overexpressed in HNSCC. No correlation was noted between CENP-F amplification and clinicopathologic parameters. However, CENP-F overexpression correlated with nodal metastasis.

PMID: 11303627

normal and aberrant expression of c-myc, in this study, where *in situ* methodologies were used to evaluate high-grade human breast cancers, gene amplification of c-myc appears to play a key role in regulating expression of its mRNA and protein.

PMID: 15083194